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Current knowledge on isobutanol production with *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium glutamicum*

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Due to steadily rising crude oil prices great efforts have been made to develop designer bugs for the fermentative production of higher alcohols, such as 2-methyl-1-butanol, 3-methyl-1-butanol and 2-Methyl-1-propanol (isobutanol), which all possess quality characteristics comparable to traditional oil based fuels. The common metabolic engineering approach uses the last two steps of the Ehrlich pathway, catalyzed by 2-ketoacid decarboxylase and an alcohol dehydrogenase converting the branched chain 2-ketoacids of L-isoleucine, L-leucine and L-valine into the respective alcohols. This strategy was successfully used to engineer well suited and industrially employed bacteria, such as *Escherichia coli*, *Bacillus subtilis* and *Corynebacterium glutamicum* for the production of higher alcohols. Among these alcohols, isobutanol is currently the most promising one regarding final titer and yield. This article summarizes the current knowledge and achievements on isobutanol production with *E. coli*, *B. subtilis* and *C. glutamicum* regarding the metabolic engineering approaches and process conditions.

Key words: *Escherichia coli*, *Bacillus subtilis*, *Corynebacterium glutamicum*, biofuel, isobutanol, Ehrlich pathway, 2-ketoacids, synthetic biology

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Higher alcohols, such as isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol, possess several beneficial characteristics, e.g., a low hygroscopicity, vapor pressure and corrosivity, full compatibility with existing engines and pipelines, and a high energy density, allowing safer handling and more convenient and more efficient use, when compared with the traditional biofuel ethanol.[1] The fermentative production of these non-natural alcohols with bacteria can be achieved by a metabolic engineering approach using the last two steps of the so-called Ehrlich pathway, i.e., a decarboxylation and subsequent reduction of branched chain 2-keto acids, which are natural intermediates of the branched chain amino acid biosynthesis in many bacteria. Besides implementation of this synthetic pathway, metabolic fine-tuning of the respective bacterial host and adjustment of the process conditions is important to obtain efficient higher alcohol production systems.

Strategies for Metabolic Engineering of *E. coli*, *B. subtilis* and *C. glutamicum* for Isobutanol Production

The common strategy to engineer *E. coli*, *B. subtilis* and *C. glutamicum* for the production of isobutanol is the implementation of the last two reactions of the Ehrlich pathway. This is accomplished by expression of genes encoding a broad range 2-ketoacid decarboxylase (KIVD; encoded by *kivd*) from *Lactococcus lactis* and an alcohol dehydrogenase (ADH) from either *Saccharomyces cerevisiae*, *C. glutamicum*, *E. coli* or *L. lactis* (encoded by *adh2*, *adhA*, *yqhD* and *adhA*, respectively).[2-6] These enzymes catalyze the conversion of the L-valine precursor 2-ketoisovalerate (KIV) to isobutanol via isobutyraldehyde (**Fig. 1**). Furthermore, for all three organisms it was shown that additional overexpression of the genes coding for acetohydroxyacid synthase (AHAS), acetohydroxyacid isomeroreductase (AHAIR), and dihydroxyacid dehydratase (DHAD) is beneficial for isobutanol production,

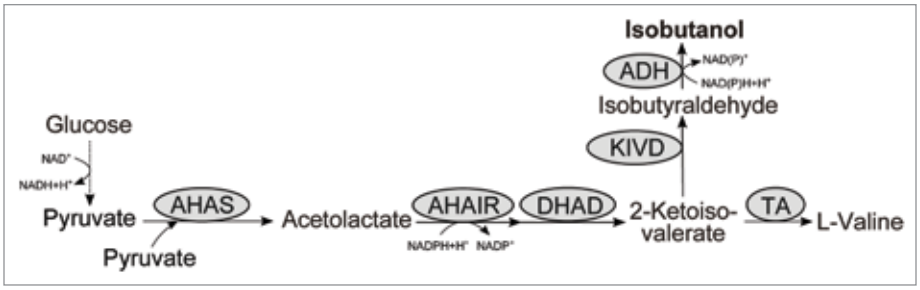

Figure 1. Enzymes of the biosynthetic pathway of L-valine and the synthetic pathway from 2-ketoisovalerate to isobutanol. ADH, alcohol dehydrogenase; AHAIR, acetohydroxy isomeroreductase; AHAS, acetohydroxyacid synthase; DHAD, dihydroxyacid dehydratase; KIVD, 2-ketoacid decarboxylase.

due to an increased drain off of pyruvate and increased KIV availability (**Fig. 1**).[2,5,6] However, since AHAS enzymes from *E. coli*, *B. subtilis* and *C. glutamicum* have lower affinities toward pyruvate compared with competing enzymes, such as pyruvate formate lyase, pyruvate dehydrogenase complex (PDHC) or lactate dehydrogenase (LDH), increasing the intracellular pyruvate availability by knockout of the respective genes is crucial for an efficient production process. Accordingly, stepwise deletion of the genes *adhE*, *ldhA*, *frdAB*, *fnr*, *pta* and *pflB* (encoding alcohol dehydrogenase, D-lactate dehydrogenase, fumarate reductase, transcriptional regulator FNR, phosphate acetyltransferase, pyruvate formate lyase, respectively) in *E. coli* JCL16/pSA55/pSA69 resulted under microaerobic conditions in a drastic increased substrate specific yield ($Y_{P/S}$) of about 0.86 mol isobutanol per mol of glucose, mainly due to increased pyruvate availability (**Table 1**).[2]

We recently engineered *C. glutamicum* for the aerobic production of L-valine and found that inactivation of the PDHC by deletion of the *aceE* gene encoding the E1p subunit of the PDHC increases pyruvate availability and led to the formation of pyruvate, L-alanine and L-valine.[7] Additional inactivation of the PQO and overexpression of the *ilvBNCE* genes, encoding AHAS, AHAIR and transaminase B (TA) in *C. glutamicum* Δ*aceE* shifted the product spectrum toward L-valine and the resulting strain *C. glutamicum* Δ*aceE* Δ*pqo* (pJC4*ilvBNCE*) produced about 225 mM L-valine with a $Y_{P/S}$ of 0.52 mol L-valine per mol glucose in fed-batch fermentations.[8] Based on these results, we subsequently engineered *C. glutamicum* for the aerobic production of KIV by inactivation of TA (encoded by *ilvE*) in *C. glutamicum* Δ*aceE* Δ*pqo* and additional overexpression of the genes coding for AHAS, AHAIR and DHAD. The resulting strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* (pJC4*ilvBNCD*) produced about 190 mM KIV with a $Y_{P/S}$ up to 0.5 mol KIV per mol of glucose[9] and thus seemed to be an excellent basis to engineer *C. glutamicum* for the production of isobutanol. However, for isobutanol production with *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* (pJC4*ilvBNCD*) under oxygen deprivation conditions, it was not only necessary to express *kivd* and *adh2*, but also to prevent L-lactate and succinate formation by deletion of the LDH and malate dehydrogenase (MDH) genes *ldhA* and *mdh*.[5]

In *B. subtilis,* the combined (over) expression of the genes encoding AHAS, AHAIR, DHAD, KIVD and ADH2 resulted in significant isobutanol formation. However, the overall $Y_{P/S}$ was only about 0.2 mol isobutanol per mol of glucose and the best producing strain *B. subtilis* UL03 (**Table 1**) secreted significant amounts of the by-products acetate, lactate and ethanol.[6] Therefore, it can be speculated that inactivation of pyruvate consuming pathways may also further improve isobutanol production with *B. subtilis*.

Isobutanol production from glucose with *E. coli, C. glutamicum* and *B. subtilis* was performed under oxygen limitation, providing not only an increased pyruvate supply but also an increased availability of reducing power (reduction equivalents). Maintaining a balanced redox state is crucial for an efficient production process under oxygen limitation. AHAIR enzymes of *E. coli* and *C. glutamicum* are NADPH-dependent, whereas different types of ADHs accept either $NADH^+H^+$ or $NADPH^+H^+$. The formation of one mol isobutanol from pyruvate requires one mol $NADH^+H^+$ and one mol $NADPH^+H^+$ or two moles $NADPH^+H^+$, respectively. Regarding that the bacteria generate two moles $NADH^+H^+$ per mol of glucose in the course of glycolysis, for efficient isobutanol production the conversion of $NADH^+H^+$ to $NADPH^+H^+$ is essential. Atsumi et al.[3] showed that the native NADPH-dependent ADH encoded by *yqhD* rather than the NADH-dependent ADH2 from *S. cerevisiae* contributes to isobutanol formation with *E. coli*. Therefore, under the conditions tested, isobutanol production was completely NADPH-dependent. As *E. coli* possesses a membrane bound transhydrogenase catalyzing the proton transfer from $NADH^+H^+$ to $NADP^+$ and since Atsumi et al.[2] reached a high $Y_{P/S}$ of 0.86 mol isobutanol per mol glucose without optimizing the $NAD(P)H^+H^+$ supply, this organism seems to be highly flexible to maintain a balanced redox state under microaerobic conditions. To circumvent the use of the energy consuming transhydrogenase reaction, recently Bastian et al.[10] engineered a fully NADH-dependent pathway for anaerobic isobutanol production with *E. coli*. By saturation mutagenesis of *ilvC* a NADH-dependent variant of AHAIR was identified showing a strong preference for $NADH^+H^+$ over $NADPH^+H^+$. Furthermore, the catalytic efficiency and the affinity toward isobutyraldehyde of the NADH-dependent ADHA from *L. lactis* was significantly improved by random mutagenesis and recombination of the useful mutations. Plasmid bound expression of the engineered genes coding for AHAIR and ADHA in *E. coli* 1993

Table 1. Relevant characteristics of selected isobutanol producing strains of *E. coli, C. glutamicum* and *B. subtilis*

Strain	Relevant characteristics	$Y_{P/S}$ [mol/mol][1]	Reference
E. coli JCL260/pSA55/ pSA69	*E. coli* Δ*adhE* Δ*ldhA* Δ*frdBC* Δ*fnr* Δ*pta* Δ*pflB* (pSA55) (pSA69); *E. coli* JCL16 with deletion of *adhE*, *ldhA*, *frdBC*, *fnr*, *pta* and *pflB*, encoding alcohol dehydrogenase (ADHE), D-lactate dehydrogenase (LdhA), fumarate reductase (FRD), transcriptional regulator FNR, phosphate acetyltransferase (PTA), pyruvate formate lyase (Pfl), respectively. Additional overexpression of *kivd* from *L. lactis* encoding 2-ketoacid decarboxylase, *adh2* from *S. cerevisiae* encoding alcohol dehydrogenase 2, *alsS* from *B. subtilis* encoding acetohydroxyacid synthase (AHAS), the *ilvCD* genes from *E. coli* encoding, isomeroreductase (AHAIR), and dihydroxyacid dehydratase (DHAD).	0.86	Atsumi, et al. 2008
E. coli 1993 (pGVferm6)	*E. coli* Δ*ldhA-fnr::FRT,* Δ*adhE::FRT,* Δ*frd::FRT,* Δ*pflB::FRT, F'(lacIq$^+$),* Δ*ilvC::PLlacO1::Ll_kivd1::Ec_ilvD_coEc::FRT,* Δ*pta::PLlacO1::Bs_alsS1, FRT ::KAN::FRT,* (pGVferm6). *E. coli* BW25113 with inactivated LdhA, ADHE, FRD and Pfl. Inactivation of AHAIR by integration of *kivd* from *L. lactis* and *ilvD* from *E. coli* under control of P_{LlacO1}. Inactivation of Pta by intergration of *alsS* under control of P_{LlacO1}. Expression of mutated *ilvC* (A71S:R76D:S78D: Q110V) from *E. coli* and *adhA* (Y50F:I1212T: L264V) from *L. lactis* under control of P_{LlacO1} on plasmid pGVferm6.	1.03	Bastian, et al. 2011
C. glutamicum Iso 7	*C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* (pJC4*ilvBNCD-pntAB*) (pBB1*kivd-adhA*); *C. glutamicum* ATCC 13032 with deletion of *aceE*, *pqo*, *ilvE*, *ldhA* and *mdh* encoding the E1p subunit of the pyruvate dehydrogenase complex, the pyruvate:quinone oxidoreductase, the transaminase B, the L-lactate dehydrogenase, and malate dehydrogenase, respectively. Additional overexpression of the *ilvBNCD* genes from *C. glutamicum* encoding AHAS, AHAIR and DHAD, the *pntAB* genes from *E. coli* encoding the membrane bound transhydrogenase, *kivd* from *L. lactis* encoding 2-ketoacid decarboxylase, and *adhA* from *C. glutamicum* encoding alcohol dehydrogenase A.	0.77	Blombach, et al. 2011
B. subtilis UL03	*B. subtilis* Δ*amyE*::(P_{43}::*kivd-adh2*-Spc^r), P_{43}::*ilvD-ilvC-alsS*-Spc^r, Em^r; *B. subtilis* 168 with the integrated genes *kivd* from *L. lactis* encoding 2-ketoacid decarboxylase, *adh2* from *S. cerevisiae* encoding alcohol dehydrogenase 2, and *ilvCD*, and *alsS* from *B. subtilis* encoding AHAIR, DHAD and AHAS, respectively. All integrated genes are under control of the strong P_{43} promoter.	0.2	Li, et al. 2011

[1]The substrate specific yield ($Y_{P/S}$) is given in mol isobutanol per mol of glucose. *E. coli* JCL260/pSA55/pSA69, *C. glutamicum* Iso7 and *B. subtilis* UL03 were cultivated under oxygen limitation in shaken flasks or bottles. *E. coli* 1993 (pGVferm6) was cultivated anaerobic in flasks.

resulted under anaerobic conditions in a maximal $Y_{P/S}$ of 1 mol isobutanol per mol of glucose (**Table 1**).[10]

Our approach with *C. glutamicum* included the expression of AHAIR and the native ADHA or the ADH2 from *S. cerevisiae* requiring in both cases one mol $NADH^+H^+$ and one mol $NADPH^+H^+$ for isobutanol production. When we inactivated MDH to avoid succinate formation and to increase precursor availability, we observed a severe reduction of glucose consumption, theoretically due to an unbalanced redox state of the cells. Consequently, we expressed the *E. coli* transhydrogenase genes *pntAB* and found that the resulting strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* (pJC4*ilvBNCD-pntAB*) (pBB1*kivd-adh2*) regained its ability to efficiently consume glucose and showed improved isobutanol production. Inactivation of the malic enzyme (MalE) gene *malE* in *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* (pJC4*ilvBNCD*)(pBB1*kivd-adh2*) and the *pntAB* expressing strain *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ilvE* Δ*ldhA* Δ*mdh* (pJC4*ilvBNCD-pntAB*)(pBB1*kivd-adhA*), in the following designated as strain Iso7 (**Table 1**), led to complete or partial abolishment of isobutanol formation, respectively. These results indicate the activity of an ATP-dependent transhydrogenase-like metabolic cycle, consisting of pyruvate and/or PEP carboxylase, MDH and MalE, contributing to the conversion of $NADH^+H^+$ and $NADP^+$ to $NADPH^+H^+$ and $NADH^+$ and thus maintaining a balanced redox state (**Fig. 2**). Such a transhydrogenase-like cycle has previously been proposed to be present,[11] although so far there was no experimental evidence for the

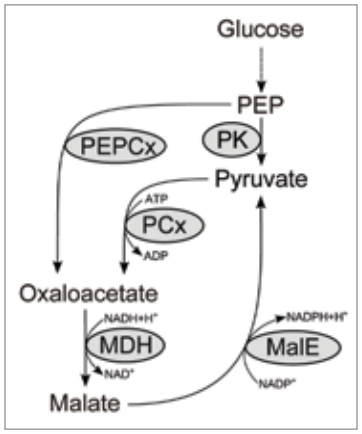

Figure 2. Proposed transhydrogenase-like cycle in *C. glutamicum*. MalE, malic enzyme; MDH, malate dehydrogenase; PCx, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCx, PEP carboxylase; PK, pyruvate kinase.

operation of this cycle in *C. glutamicum*. However, the overexpression of *malE* in our producer strains might be a possibility to further improve isobutanol production with *C. glutamcum*.[5]

Smith et al.[4] also engineered *C. glutamicum* for the production of isobutanol and tried to increase $NADPH^+H^+$ availability by inactivation of the glucose 6-phosphate isomerase gene to redirect the carbon flux into the $NADPH^+H^+$ generating pentose phosphate pathway. Unfortunately, this attempt to improve isobutanol production failed, probably due to an imbalance in the redox state of the cell.

Taken together, the results with *E. coli*, *C. glutamicum* and *B. subtilis* demonstrate that the implementation of the last two steps of the Ehrlich pathway is a useful and for several hosts compatible synthetic metabolic engineering approach. However, besides optimizing the metabolic pathway from pyruvate to isobutanol, increasing pyruvate and $NAD(P)H^+H^+$ supply is essential to design efficient bugs for the production of isobutanol under oxygen limitation.

Process Conditions for Isobutanol Production

The isobutanol production performance of the *E. coli* and *C. glutamicum* strains described above was first analyzed in slowly shaken flasks or bottles under oxygen limitation.[2,5] For the most promissing strains fed-batch processes were developed, differing in the conditions applied. For *E. coli* JCL260/pSA55/pSA69 strictly aerobic conditions were used, with in situ product removal by gas stripping with air and subsequent sampling of isobutanol by condensing.[12] This process resulted in isobutanol titers of more than 50 g/l (i.e., 675 mM) with a $Y_{P/S}$ of 0.68 mol isobutanol per mol of glucose within 72 h, yielding a productivity of about 0.7 $gl^{-1}h^{-1}$ (i.e., 9.4 $mmoll^{-1}h^{-1}$) at a cultivation temperature of 30°C.[12] However, in spite of aerobic conditions during the whole process, cells stopped growing after 10 h, reaching a maximum cell density of 6.7 g/l, probably due to isobutanol toxicity. Increasing the temperature from 30°C to 37°C to increase the vapor pressure for a more efficient stripping process, failed as *E. coli* JCL260/pSA55/pSA69 showed at 37°C a drastically reduced final isobutanol titer and yield.[12] Thus, the isobutanol-induced growth arrest limits the overall productivity for an industrial scale application. To overcome isobutanol toxicity, Atsumi et al.[13] recently isolated an isobutanol-tolerant *E. coli* strain by a sequential transfer method. However, the final strain was more tolerant to isobutanol, but showed much lower isobutanol formation, when compared with the parental strain *E. coli* JCL260/pSA55/pSA69.[13] More recently, Minty et al. used experimental evolution combined with genome resequencing to identify the genotypic adaptations of *E. coli* lineages with increased isobutanol tolerance. Thereby, the authors identified a set of mutations (*marC*, *hfq*, *mdh*, *acrAB*, *gatYZABCD*, *rph*) common in several isobutanol tolerant lineages and they speculated that *rpoS* and post-transcriptional regulators such as *hfq* are promising targets to improve isobutanol production with *E. coli*.[14]

Since we observed that the best *C. glutamicum* producer (strain Iso7) showed under aerobic conditions a more than 2-fold lower $Y_{P/S}$ (unpublished results), we tried to transfer the process conditions from the bottle to a bioreactor and thus developed a two phase fermentation. *C. glutamicum* Iso7 was cultivated in the first phase under aerobic conditions. In this phase the cells grew with glucose and acetate to high cell densities and produced no isobutanol. After complete consumption of the acetate, the cells stopped growing and the production phase was started by switching off aeration. The residual oxygen in the culture was rapidly consumed and *C. glutamicum* Iso7 produced up to 180 mM isobutanol with a volumetric productivity of about 4.4 $mmoll^{-1}h^{-1}$.[5] The reasons for the production stop at 180 mM isobutanol remain unclear but they might also be attributed to isobutanol toxicity for the cells. The negative effect might be overcome by developing an integrated stripping process with nitrogen, which has been successfully applied for 1-butanol production with Clostridia.[15]

A significantly reduced $Y_{P/S}$ was observed in the fed-batch fermentations with *C. glutamicum* Iso7 when compared with the cultivations in shaken bottles.[5] However, the differential $Y_{P/S}$ of *C. glutamicum* Iso7 was constant in the whole production phase and therefore this effect can hardly be explained by isobutanol toxicity, but indicates that the physiological state of the cell during the transition from aerobic to oxygen-deprived conditions may have an influence on the overall production behavior.[5] Recently, Martínez et al.[16] investigated the role of the transition from aerobic to anaerobic conditions in a succinate production process with *E. coli* and showed that introducing a microaerobic phase at the end of the aerobic growth phase led to an adjustment of the enzymatic machinery and to improved succinate production under anaerobic conditions. This, in consequence, opens the possibility to improve our *C. glutamicum* production process by introducing microaerobic conditions at the end of the aerobic growth phase. However, the physiological changes of *C. glutamicum* during the transition from aerobic to anaerobic conditions have so far not been investigated. A deep insight in the metabolic adaptation of the cell to such alternating culture conditions will help to further optimize isobutanol production by novel metabolic engineering approaches and applying optimal process conditions.

Outlook

Successful metabolic engineering approaches are available to transform bacterial hosts such as *E. coli*, *B. subtilis* and *C. glutamicum* into efficient isobutanol producers. However, to reach high isobutanol productivities and titers, overcoming isobutanol toxicity is indispensable in the up-scaling process, aside from optimizing the process conditions. Isobutanol toxicity might be overcome by integrated product removal and/or the use of highly tolerant strains. In addition, environmentally friendly bioprocesses require the use of second generation feedstocks, such as lignocellulose, which do not compete for feed and food. Therefore, expanding the substrate spectrum (for e.g., to pentoses) of relevant designer bugs is a prerequisite for a bio-based production process of higher alcohols and therefore a most relevant goal for future studies.

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